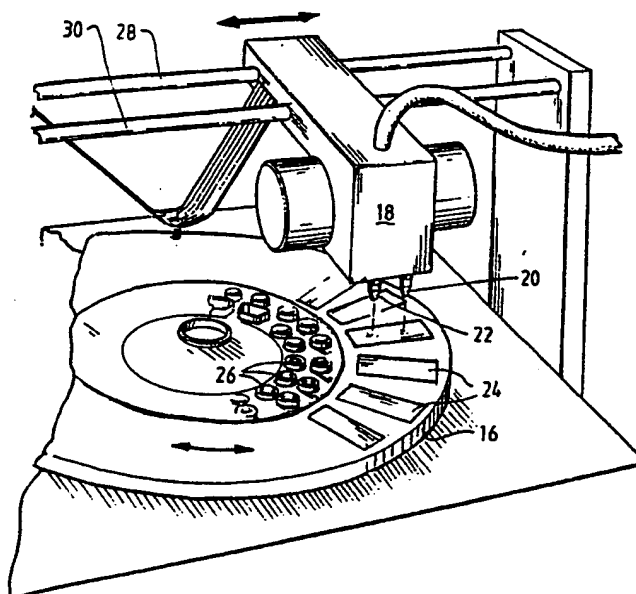




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵: G01N 1/28, 35/00, B05B 1/34	A1	(11) International Publication Number: WO 92/01919 (43) International Publication Date: 6 February 1992 (06.02.92)
(21) International Application Number: PCT/AU91/00170 (22) International Filing Date: 29 April 1991 (29.04.91) (30) Priority data: PK 1231 18 July 1990 (18.07.90) AU (71) Applicant (for all designated States except US): AUSTRALIAN BIOMEDICAL CORPORATION LTD. [AU/AU]; 96 Rickets Road, Mount Waverley, VIC 3149 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only): HEALEY, Kevin [AU/AU]; 12 Tilba Street, Essendon, VIC 3040 (AU). BAXTER, Graham, Leslie [AU/AU]; 76 Gillies Street, Fairfield, VIC 3078 (AU). (74) Agent: SMITH SHELSTON BEADLE; 207 Riversdale Road, Hawthorn, VIC 3122 (AU).		(81) Designated States: AT (European patent), AU, BE (European patent), BG, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), + NO, PL, RO, SE (European patent), SU, US. Published <i>With international search report.</i>

(54) Title: AUTOMATIC TISSUE STAINING FOR IMMUNOHISTOCHEMISTRY



(57) Abstract

Apparatus (10) for the automatic staining of tissue includes a body (12) on which a carousel (16) rotates. The carousel (16) is able to carry a number of slides (24) bearing tissue samples. A delivery head assembly (18) is mounted on the body (12) for movement across the diameter of the carousel (16). The head assembly contains a clear nozzle (20) and a spray nozzle (22). A version (50) of the spray nozzle (22) for spraying a fluid biochemical agent onto a slide (24) has a main body (52) a plug (56) in the main body (52) and a cap (54) on the main body (52) between which there is a swirl chamber (68) into which fluid passes from the body (52). The fluid enters the swirl chamber (68) such that flow in the chamber (68) is concentric to the chamber axis and thereafter flows from the body (52) through an exit (66), thus minimising damage to the biochemical agent.

+ DESIGNATIONS OF "SU"

It is not yet known for which States of the former Soviet Union any designation of the Soviet Union has effect.

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AUTOMATIC TISSUE STAINING FOR IMMUNOHISTOCHEMISTRY

This invention relates to immunohistochemistry, and in particular relates to apparatus for automatically staining tissue sections, or cell preparations.

Histochemistry is a branch of biochemistry devoted to the study of the chemical composition and structure of animal and plant tissues. It involves the use of microscopic, x-ray diffraction and radioactive tracer techniques in examining the cellular composition and structure of bones, blood, muscle and other animal and vegetable tissues.

Immunohistochemistry is useful for directly viewing the cellular distribution of a molecule (marker) using labelled antibodies or other ligands including nucleic acid probes. Labels include enzymes, radioisotopes and fluorescent molecules. The technique can be applied to whole cells, for example for identification of lymphomas (white blood cell cancers) or to tissue sections, for example for cancer diagnosis.

The specific (or primary) antibody may be labelled directly. Alternatively and more often, a second antibody carrying the label is used to specifically bind to the first. Also, the tissue may require pretreatment to reveal the marker of interest (enzymic) or to remove non-specific effects.

A number of methods have been developed to amplify the visual signal and this may add several steps to the technique. Ultimately, in the case of an enzyme-label a substrate is applied which produces a coloured product at the site of the label. The surrounding tissue is then counterstained to provide contrast. Therefore most protocols for carrying out staining involve a large number of incubations of various time periods, separated by washing to remove spent reagents.

Each operation requires considerable care in the application of small amounts (50 to 200 microlitres) of reagents to cover the tissue adequately and also in the washing steps to ensure complete removal of spent reagents and to avoid accidental removal of tissue from the slide. Thus, when a large number of slides are involved the procedure is labour intensive, tedious and can suffer from a lack of reproduc-

ibility.

A manual technique currently utilised for staining tissue involves a skilled technician performing all operations manually.

Firstly, glass specimen slides are supported on trays. When staining is carried out above ambient temperature, heating is normally provided using a specially designed temperature-controlled template with provision to allow high humidity.

The slides are washed with a buffer stream from a hand-operated dispensing bottle. The slides are then cleared of liquid by being set vertically to drain, and wiped around the specimen with paper towel material.

The biochemical agent delivery is via a manual pipettor positioned by eye such that the fluid is spread to cover the tissue sample. Chemical reagents with short active lives are manually mixed in vials using standard pipettors.

The control of event sequences and times is performed manually with the aid of a stopwatch and note pads.

It is obvious that such a manual process is inherently inaccurate, time consuming and costly.

There have been prior attempts to automate such a process, and details of such attempts appear hereunder.

(1) Stross, W.P., Jones, M., Mason, D.Y.

J. Clin. Pathol. Jan 1989 42(1) p 106-112

These authors have modified an existing tissue processing instrument (Histokinette E7326; British American Optical Corporation) to carry out immunohistochemical staining of tissues in a semi-automated manner. Slides are placed in racks which are dipped into tanks of reagents which are used repeatedly for up to four months. The method was only applied to the (APAAP) Alkaline phosphatase staining method and did not automate the application of primary antibody or substrate.

(2) Brigati, D.J., Budgeon, L.R., Unger, E.R., Koebler, D.,

Cuomo, C., Kennedy, T., Perdomo, J.M.

J. Histotechnol. 11(3), 1988 p 165-183

These authors claim to have developed the first automated method for immunocytochemistry. The method uses a triaxial

robotic slide system to move racks of slides between reagents. The slides are paired so that reagents fill the gaps between slides by capillary action. The system is claimed to be able to carry out the complete immunostaining procedure as well as in-situ hybridisation and has been commercialised by Fischer Scientific Co. (U.S.A.).

- (3) Stark, E., Faltinat, D., Von der Fecht, R.,
J. Immunological Methods 107 (1988) p 89-92

These authors have described an instrument in which up to 30 slides are carried on a carousel which can be rotated rapidly to remove reagents. The antibodies or other solutions are pipetted onto the slides by standard plastic syringes.

This approach has a major disadvantage in that it uses large volumes of expensive reagents and does not automate the primary antibody step.

- (4) Mehven, L., Med. Lab. World Feb 1989 p 45-46

Described a novel coverslip device which is used to create a capillary gap between the slide and coverslip.

Reagents are transferred from vials in a carousel to a funnel part of the coverslip using an automatic pipette and an x-slide device. 10 basic method programs can be used to run 20 slides automatically with a variety (up to 9) primary antibodies. The instrument has been commercialised by Shandon Scientific (U.K.) under the "Cadenza" trade name.

- (5) Recently Lipshaw (U.S.A.) have released an instrument for the automatic staining of batches of slides by the peroxide technique. The system involves the transfer of a rack of slides between baths of reagents with a robotic arm. The instrument is limited to use for slides undergoing identical methods, is not useful for adding primary antibody and uses large volumes of other reagents.

It is an object of the present invention to provide improved apparatus for immunohistochemical staining tissue or cell preparations.

The invention provides apparatus for immunohistochemical sample processing, including sample support means, washing

means for dispensing washing fluid onto said sample, clearing means for cleaning said sample, and agent dispensing means for dispensing an agent onto said sample.

The invention also provides apparatus for processing tissue samples in immunohistochemistry, including slide support means for supporting at least one slide, said slide support means being constituted by a rotatable carousel, and head assembly means adapted to move relative to said slide support means, and adapted to dispense fluid to a slide on said support means.

The invention further provides a device for spraying a surface with fluid, said device including a body having a bore therethrough for the passage of said fluid, means located in said body for changing the flow of said fluid from a direction generally along said bore to a generally annular flow, there being a swirl chamber located in proximity to a fluid exit, said swirl chamber being adapted to turn said generally annular flow to a generally concentric flow prior to fluid leaving said chamber through said fluid exit.

A preferred embodiment of the invention, will be described in detail hereinafter with reference to the accompanying drawings, in which:-

Fig. 1 is a perspective view of automatic tissue staining apparatus;

Fig. 2 is a perspective view of a detail of apparatus generally similar to that of Fig. 1;

Fig. 3 is a front elevation of a clear nozzle;

Fig. 4 is a side elevation of the nozzle of Fig. 3;

Fig. 5 is a rear elevation of part of the nozzle of Fig. 3;

Fig. 6 is an underneath plan view of the nozzle of Fig. 3;

Fig. 7 is a section along the lines A-A of Fig. 3;

Fig. 8 is a side elevation of a spray nozzle;

Fig. 9 is an end elevation of a spray nozzle body;

Fig. 10 is a section along the lines A-A of Fig. 9;

Fig. 11 is a section along the lines B-B of Fig. 10;

Fig. 12 is a perspective view of a spray nozzle end cap;

Fig. 13 is a front elevation of the cap of Fig. 13;

Fig. 14 is a partially sectioned side elevation of the cap of Fig. 13;

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Fig. 15 is a rear elevation of the cap of Fig. 13;

Fig. 16 is an end elevation of a plug; and

Fig. 17 is a side elevation of the plug of Fig. 16.

Referring firstly to Figs. 1 and 2, the automatic tissue stainer 10 includes a body 12 having a hinged portion 14 in an open position showing a horizontal carousel 16, a delivery head assembly 18, clear nozzle 20 and spray nozzle 22.

As shown in Fig. 2 - which represents a slightly different model to that illustrated in Fig. 1 - the carousel 16 is adapted to rotate about a generally vertical axis, and is further adapted to carry slides 24 on a single level surface near its periphery, and reagent or the like containers 26 towards the axis of the carousel 16.

Delivery head assembly 18 is adapted to move across the diameter of carousel 16, on rails 28 and 30, and it can be seen that a combination of rotational movement of the carousel and translational movement of the head 18 enables nozzles 20, 22 to direct material onto any part of any slide 24 or to any container 26. Preferably a third (wash fluid delivery) nozzle is also mounted on head assembly 18, and preferably each nozzle is capable of vertical movement relative to the assembly.

The apparatus 10 enables automatically controlled sequences to be carried out using various electro-mechanical systems (not shown). The apparatus 10 is operated and controlled by a keypad 32 and display 34.

The carousel 16 is adapted to be heated, preferably from beneath and possibly utilising hot air. Preferably, use is made of heated water located beneath the slide, which are located upon supports. Preferably, automatic temperature and control, of the water, is provided.

Head assembly 18 may also include a slide wash facility (not shown) which involves the delivery of a stream of buffer or wash liquid from a dispenser, in a controlled fashion, from the aforementioned third nozzle to a slide 24 located beneath the dispenser on carousel 16. Preferably, the head 18 moves along the slide axis, to evenly distribute the liquid on the slide. Preferably, the buffer liquid is supplied from a pressurised storage bottle with a valved on/off control.

The head assembly 18 also includes a slide clear facility, which is used to clear a slide 24 of liquid dispensed as described in the preceding paragraph. To so clear the slide, the nozzle 36 of Figs. 3 to 7 inclusive is utilised, which nozzle 36 may take the place of generalised nozzle 20 of Fig. 1.

Clear nozzle 36 includes a body 38 with a widened dispensing end 40 over which is located a plate 42. There is above 44 in body 36, communicating with a plenum 46 in end 40. An exit orifice 48 directs a "curtain" of air onto a slide 24. Preferably, air from a pressurised manifold is supplied to nozzle 36.

Nozzle 36 is moved by head 18 along the axis of slide 24, and the stream or curtain of air from orifice 48 pushes any fluid on the surface of slide 24 along the axis and off the sides and ends of the slide.

Head 18 also includes a biochemical agent delivery facility, using the spray nozzle 50 of Figs. 8 to 17 inclusive, which nozzle 50 may take the place of generalised nozzle 22 of Fig. 1.

Spray nozzle 50 includes a main body 52, an end cap 54 and a plug 56. Main body 52 has a knurled portion 58 adapted to be used to secure body 52 to end cap 54. Body 52 also has flow-splitting channels 60 and a ferrule seal 62, the latter for connection to a vacuum source, a pipettor or the like for operating the spray nozzle.

End cap 54 has an internal circumferential lip 64 which seals with main body 52. The cap has an axially-located exit orifice, behind which is a swirl chamber 68.

In Fig. 13 (rear elevation) is shown a structure 70 which acts to direct fluid (see the arrows) into the swirl chamber 68 to ensure that the flow is concentric to the chamber axis.

Plug 56 has a spigot 72 and a locating boss 74.

In the assembled nozzle 50 of Fig. 8, the direction of fluid is shown by arrows. The nozzle 50 achieves a small vortex spray chamber effect where fluid is forced under pressure into a circular chamber (68) in such a manner that the flow is concentric to the axis. The spray exits through

central outlet 66.

The structure and operation of the spray nozzle 50 is such that antibodies - as a reagent sub-group - may be sprayed onto a surface without loss of activity.

The proteins in antibodies are susceptible to shear forces normally encountered in conventional spray nozzles. This results in the antibodies being denatured and to consequently lose activity. For that reason, spraying of antibodies has not been considered possible.

It appears that when using the spray nozzle 50, the fluid path is such that antibodies emerge unscathed from the exit 66.

It is preferable to minimise spray head "dead space" and to optimise the fluid velocity and flow so that the correct spray section and pattern are provided to cover an area of a slide 24 from the delivery position. Preferably the area is less than the total area of the slide, more preferably in the range $1/4$ to $3/4$ of the total slide area, and more preferably $1/3$ the total slide area, and the nozzle 50 is preferably located between 10mm and 100mm above slide 24, more preferably about 30mm above a slide 24.

Furthermore, it is preferable that the nozzle 50 perform with a total fluid volume per spray of between 50 and 200 microlitres. This small volume is selected to cover the slide area with a consistent layer of fluid.

The various parts (52,54,56) of nozzle 50 are preferably formed from injection-moulded plastics material, and the nozzle is preferably connected via ferrule 62 to the delivery conduit of a pipettor delivery head. The pipettor is preferably an electrical/electronic pipettor system (not shown) which acts on a syringe to draw fluid from a container 26 (storage vial) to deliver the fluid via nozzle 50 to slide 24.

The pipettor facilitates the obtention of fluid by a controlled vertical motion whereby it moves down such that the syringe is within the fluid storage vial 26; the pipettor then operates to draw the required amount of fluid into the system; and the syringe moves up in a controlled manner to withdraw from the storage vial 26.

Some chemical agents require mixing just prior to use; they

have short active lifetimes after mixing. These items may be mixed by the use of the pipettor system drawing up one agent from its storage vial and dispensing it into the storage vial in which the second agent is held. The mixed solution is then drawn up and dispensed onto the slides as described.

As described earlier, the apparatus 10 provides an automatic tissue-staining or slide washing/drying coating process.

Preferably, there are six modes of operation:

Process Mode:	Unit automatically running program.
Load Mode:	Operator loading slides/reagents with unit prompt.
Program Mode:	Operator setting up a specific protocol program for use in process mode.
Unload Mode:	Cycle complete, operator unloads processed slides.
Self Clean Mode:	Unit self flushing the working surfaces.
Self Test Mode:	Unit running pre-run checks.

The apparatus is capable of performing any combination of the programmed protocols up to a limit of ten. The apparatus is capable of processing up to twenty slides.

Preferably, the apparatus carries a power failure battery back up to enable processing to be satisfactorily shut down at an appropriate stage.

It can be seen that this invention provides apparatus which enables the old manual techniques to be replaced by automatic procedures, operated by a less skilled operator.

CLAIMS

1. Apparatus for immunohistochemical sample processing, including sample support means, washing means for dispensing washing fluid onto said sample, clearing means for cleaning said sample, and agent dispensing means for dispensing an agent onto said sample.
2. Apparatus according to claim 1, wherein said sample is a sample located on a slide.
3. Apparatus according to claim 2, wherein said sample support means is a rotatable carousel having a flat surface for supporting said slide.
4. Apparatus according to any preceding claim, wherein said washing means, said clearing means and said agent dispensing means are located on a head assembly which is movable relative to said support means.
5. Apparatus according to claim 4, wherein said clearing means includes a nozzle adapted to deliver a curtain of air to said slide.
6. Apparatus according to claim 4, wherein said agent dispensing means is associated with a pipettor system.
7. Apparatus according to any preceding claim, wherein said agent is a reagent or biochemical agent.
8. Apparatus for processing tissue samples in immunohistochemistry, including slide support means for supporting at least one slide, said slide support means being constituted by a rotatable carousel, and head assembly means adapted to move relative to said slide support means, and adapted to dispense fluid to a slide on said support means.
9. A device for spraying a surface with fluid, said device including a body having a bore therethrough for the passage of said fluid, means located in said body for changing the flow of said fluid from a direction generally along said bore to a generally annular flow, there being a swirl chamber located in proximity to a fluid exit, said swirl chamber being adapted to turn said generally annular flow to a generally concentric flow prior to fluid leaving said chamber through said fluid exit.
10. A device according to claim 9, wherein said body

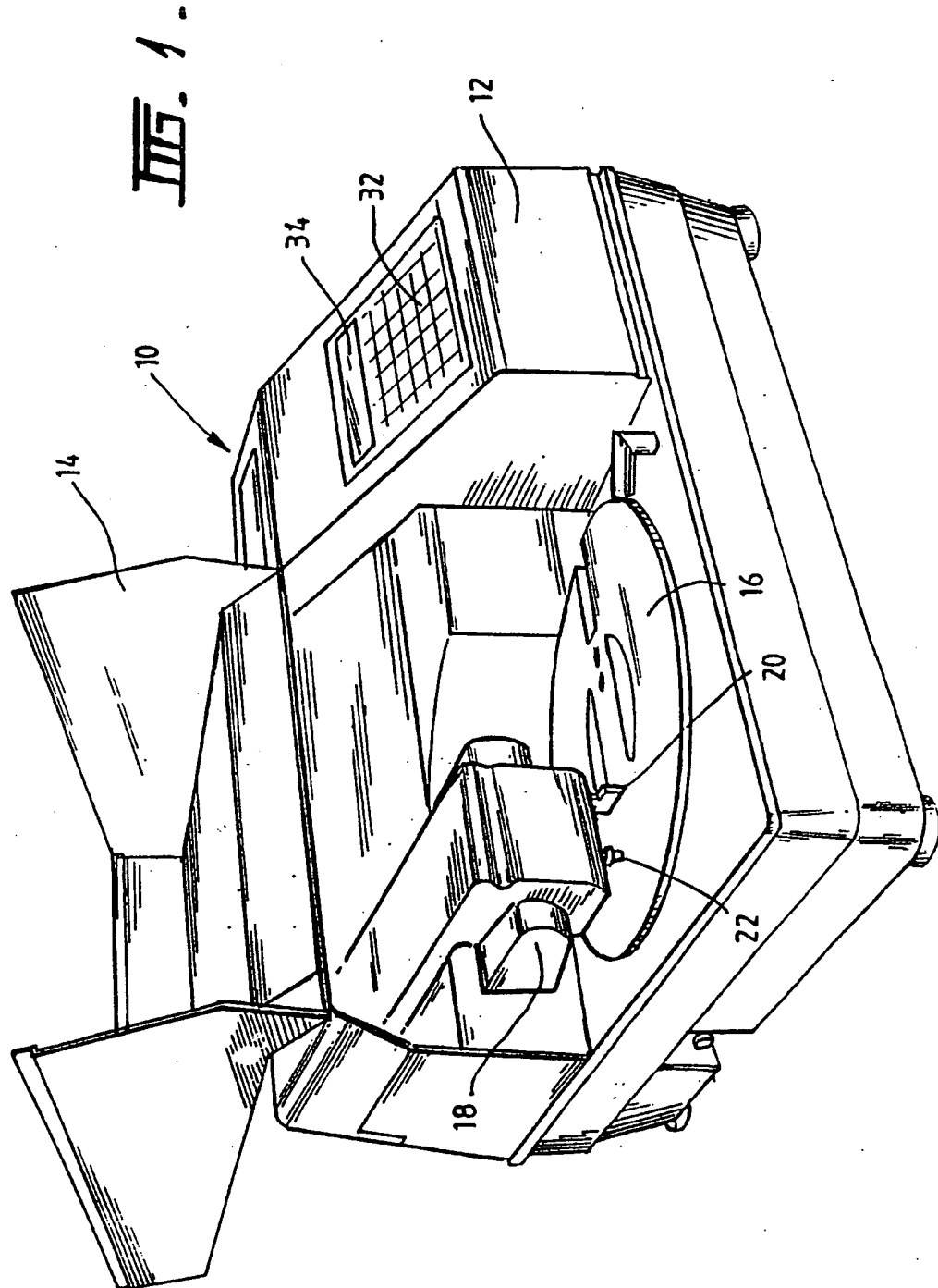
- 10 -

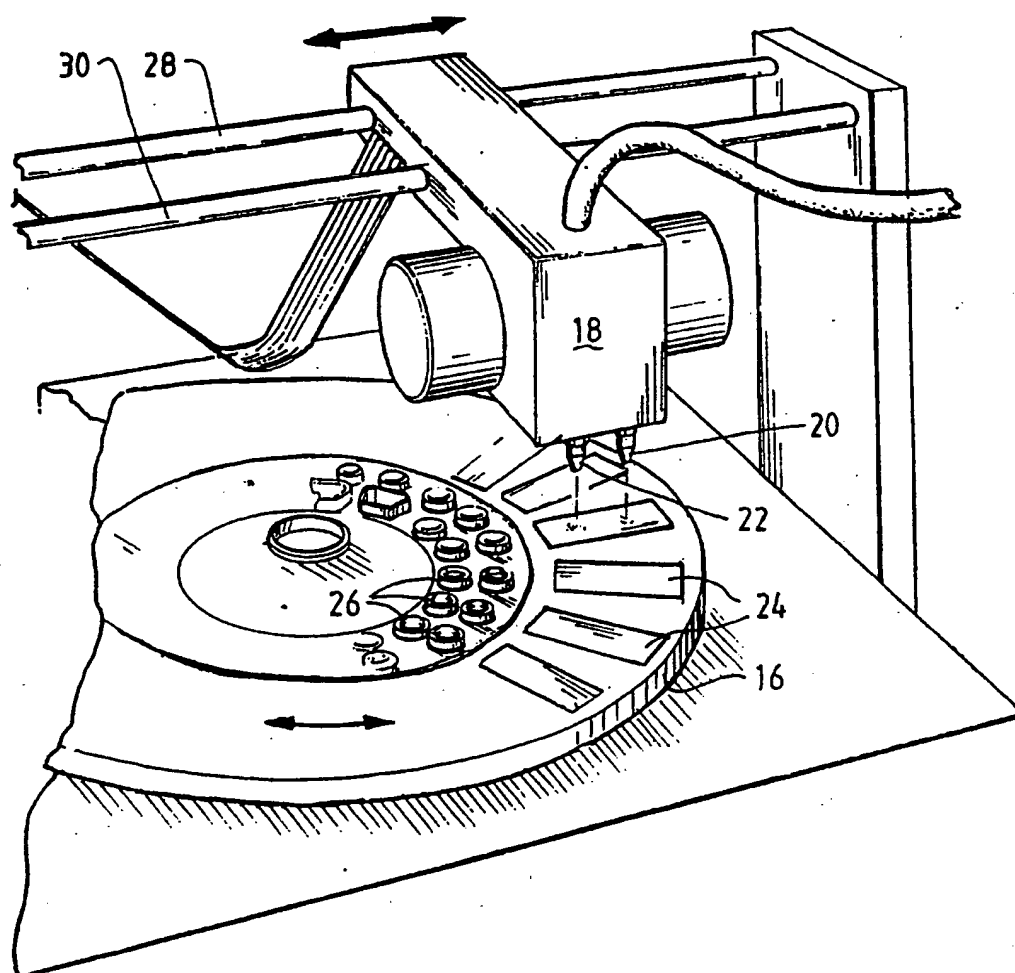
includes a plug means adapted to successively produce an increased-diameter annular flow, and means to direct said annular flow into said swirl chamber to produce said generally concentric flow.

11. A device according to claim 9 or claim 10, wherein said fluid exit is co-axial with said swirl chamber.

12. A device according to any one of claims 9 to 11, wherein said directing means is at least one channel adapted to intercept said annular flow and deliver fluid from that flow at a tangent to said chamber periphery.

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FIG. 2.

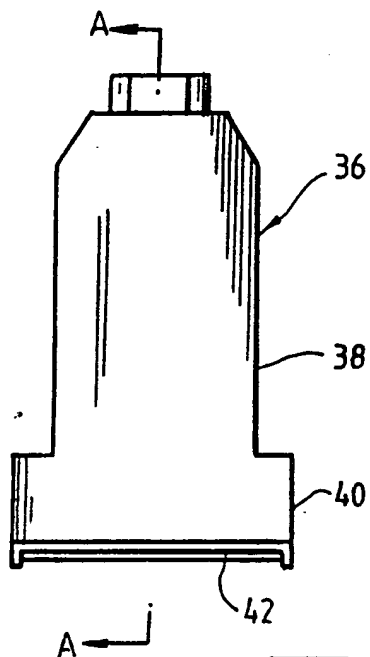


FIG. 3.

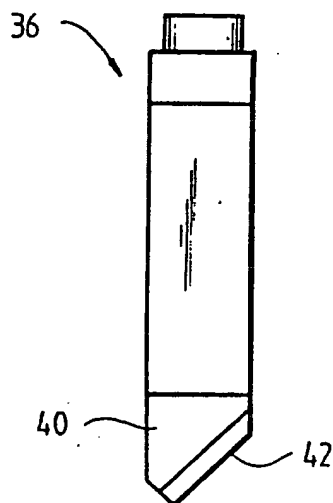


FIG. 4.

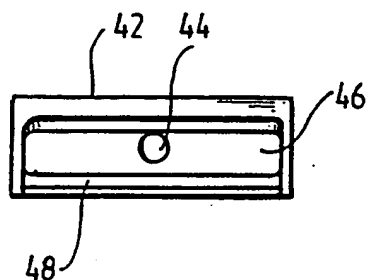


FIG. 5.

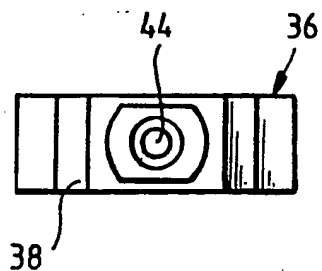


FIG. 6.

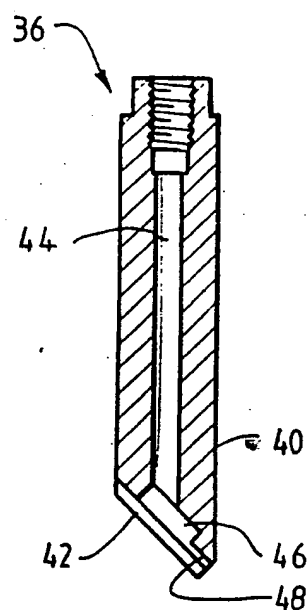


FIG. 7.

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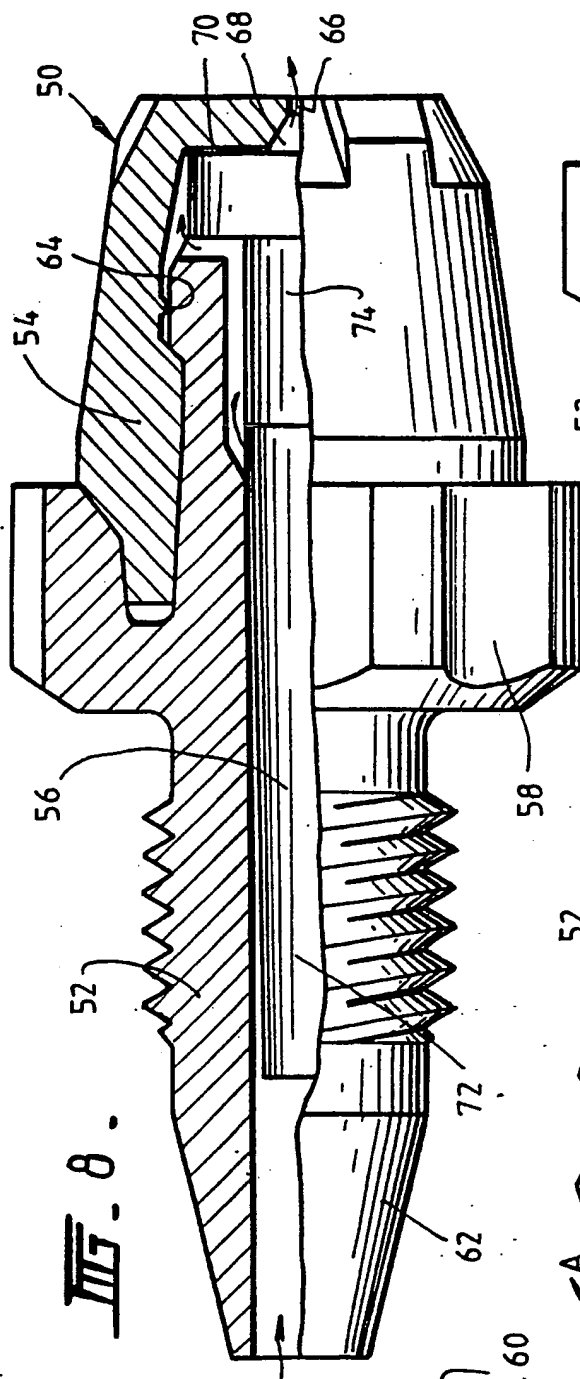


FIG. 8.

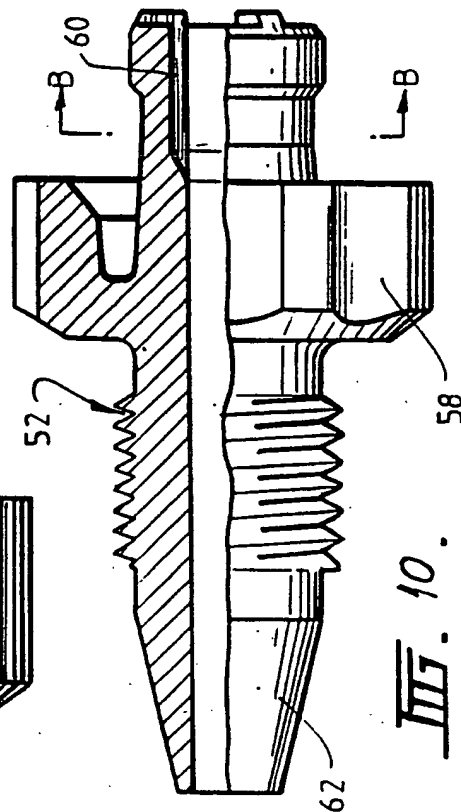


FIG. 10.

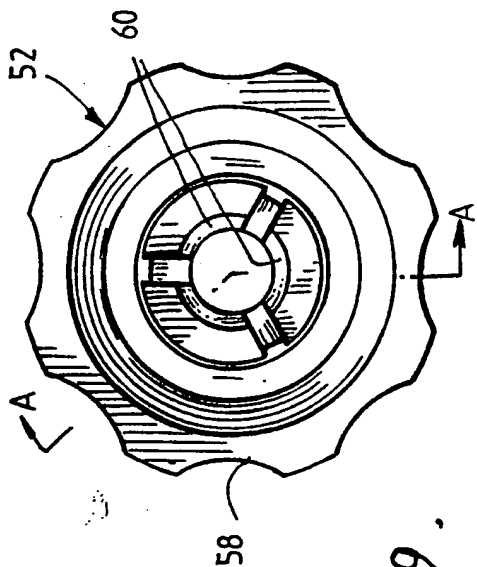


FIG. 9.

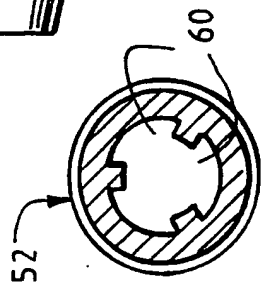


FIG. 11.

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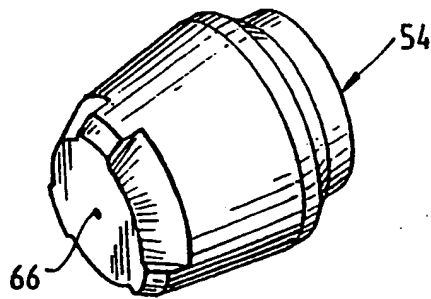


FIG. 12.

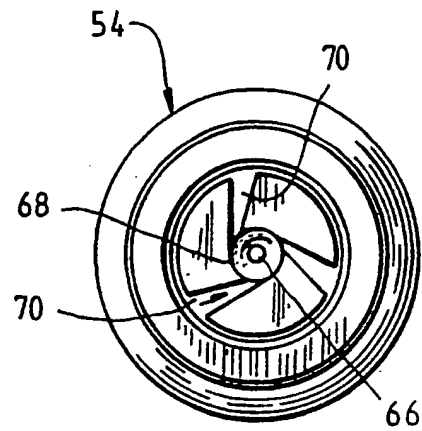


FIG. 13.

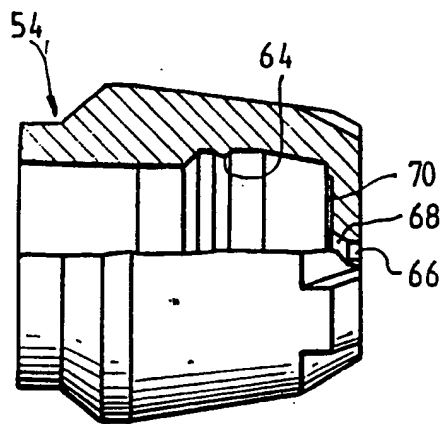


FIG. 14.

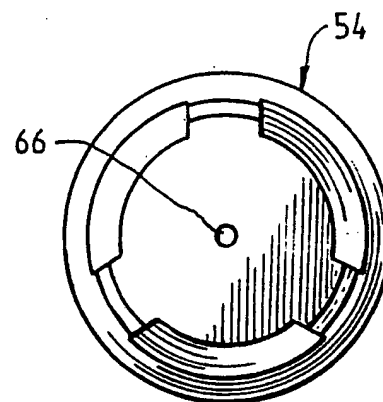


FIG. 15.

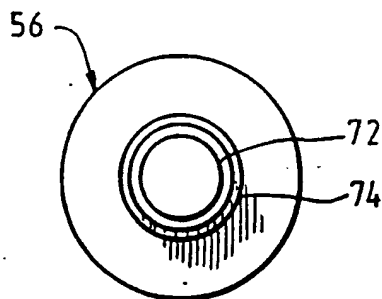


FIG. 16.

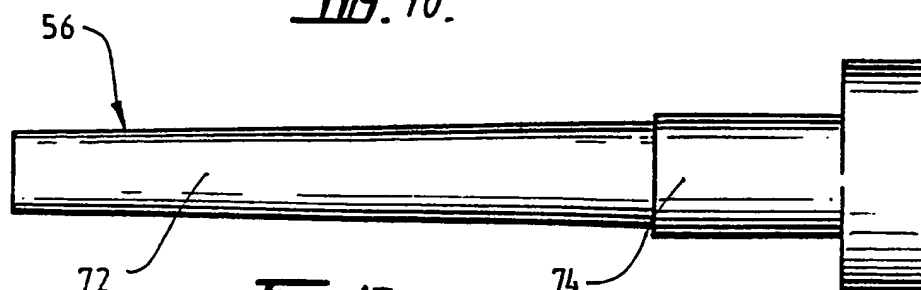
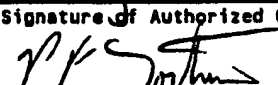


FIG. 17.

I. CLASSIFICATION SUBJECT MATTER (if several classification symbols apply, indicate all) 6		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int. Cl. ⁵ G01N 1/28, 35/00; B05B 1/34		
II. FIELDS SEARCHED		
Minimum Documentation Searched 7		
Classification System	Classification Symbols	
IPC	G01N 35/00, 35/02; B05B 1/34	
Documentation Searched other than Minimum Documentation to the extent that such documents are included in the fields searched 8		
AU : IPC as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT 9		
Category*	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X	US,A, 3368872 (NATELSON) 13 February 1968 (13.02.68) See Col 2	(1;7)
Y	line 66 - Col 3 line 27	(4)
X	US,A, 3574064 (BINNINGS et al) 6 April 1971 (06.04.71) See Col 3	(1-3,7)
Y	line 74 - Col 4 line 29, Col 4 line 74 - Col 5 line 18	(4)
X	US,A, 4837159 (YAMADA) 6 June 1989 (06.06.89) See Col 4 line 36 -	(1,7)
Y	Col 5 line 22	(4,8)
X	US,A, 4847208 (BOGEN) 11 July 1989 (11.07.89) See Col 5 lines 1-33	(1-2,7)
Y		(4,6,8)
Y	DE,A, 3805808 (EUROPAISCHES LABORATORIUM FÜR MOLEKULARBIOLOGIE) 7 September 1989 (07.09.89) See Fig 1	(4,6)
(continued)		
<p>* Special categories of cited documents: 10</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
24 June 1991 (24.06.91)	2 July 1991	
International Searching Authority	Signature of Authorized Officer	
Australian Patent Office		

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	DE,A, 908500 (BREINL et al) 8 April 1954 (08.04.54) See Figs 1,2	(9-12)
X	US,A, 4613079 (MANS) 23 September 1986 (23.09.86) See Col 2 line 38 - Col 3 line 15	(9-12)
X	GB,A, 1366581 (DELANE MANUFACTURING CO.) 11 September 1974 (11.09.74) See page 2 lines 31-99	(9-12)
X Y	US,A, 4801093 (BRUNET et al) 31 January 1989 (31.01.89) See Col 2 line 43 - Col 3 line 11	(9,11-12) (10)
X Y	CH,A, 421009 (ESB ELEKTROSTATISCHE SPRITZ-U. BEFLOCKUNGSGESELLSCHAFT G.F. VOHRINGER & CO. OHG) 31 March 1967 (31.03.67) See Figs 1-3	(9,11-12) (10)

(continued)

V. [] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

- 1.[] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:
- 2.[] Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
- 3.[] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (B):

VI. [] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

- 1.[] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
- 2.[] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
- 3.[] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. [] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- [] The additional search fees were accompanied by applicant's protest.
[] No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	DE,A, 829;040 (BORGER) 21 January 1952 (21.01.52) See Figs 1,2	(9,11-12) (10)
X Y	FR,A, 948056 (TUSCHER) 21 July 1949 (21.07.49) See Figs 1-3	(9,11-12) (10)
X Y	Patents Abstracts of Japan, C-231, page 134, JP,A, 59-49860 (MATSUSHITA DENKI SANGYO K.K.) 22 March 1984 (22.03.84)	(9,11-12) (10)
Y	Patents Abstracts of Japan, C-25, page 23 , JP,A, 55-84568 (ASAHI OKUMA SANGYO K.K.) 25 June 1980 (25.06.80)	(10)

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL APPLICATION NO. PCT/AU 91/00170

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Members			
US	3574064	CA	924619	CA	924620
		FR	2008155	GB	1274873
		GB	1278753	JP	50009317
		SE	377618	SE	385051
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US	4613079	CA	1254807	DE	3568856
		JP	6119910	EP	188060
US	4801093	AT	32498	DE	3469344
		ES	289065	FR	2547737
				EP	131501
				JP	60085759

END OF ANNEX